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The Mammalian Golgi Regulates Numb Signaling in Asymmetric Cell Division by Releasing ACBD3 during Mitosis

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SUMMARY

Mammalian neural progenitor cells divide asymmetrically to self-renew and produce a neuron by segregating cytosolic Numb proteins primarily to one daughter cell. Numb signaling specifies progenitor over neuronal fates but, paradoxically, also promotes neuronal differentiation. Here we report that ACBD3 is a Numb partner in cell-fate specification. ACBD3 and Numb proteins interact through an essential Numb domain, and the respective loss- and gain-of-function mutant mice share phenotypic similarities. Interestingly, ACBD3 associates with the Golgi apparatus in neurons and interphase progenitor cells but becomes cytosolic after Golgi fragmentation during mitosis, when Numb activity is needed to distinguish the two daughter cells. Accordingly, cytosolic ACBD3 can act synergistically with Numb to specify cell fates, and its continuing presence during the progenitor cell cycle inhibits neuron production. We propose that Golgi fragmentation and reconstitution during cell cycle differentially regulate Numb signaling through changes in ACBD3 subcellular distribution and represent a mechanism for coupling cell-fate specification and cell-cycle progression.

INTRODUCTION

Cell-fate determination is often precisely coordinated with cell-cycle progression. This is most apparent during an intrinsically asymmetric cell division, which produces two daughter cells that are already different at birth. In *Drosophila melanogaster,* for example, sensory organ precursor (SOP) cells divide asymmetrically to produce a IIA and a IIB cell (Gho et al., 1999). *Drosophila* Numb (d-Numb), a cytosolic signaling protein, distributes symmetrically during interphase but becomes localized to only one-half of the cell membrane after the SOP cell enters mitosis and, consequently, is segregated primarily into the IIB cell (Rhyu et al., 1994). This asymmetric d-Numb distribution is essential for distinguishing the two daughter cells; d-Numb loss causes both daughter cells to become IIA, whereas its symmetric inheritance yields two IIB cells. Interestingly, d-Numb loses its ability to specify cell fates shortly after mitosis; although the IIA fate requires its initial absence, newly synthesized d-Numb later accumulates in IIA cells, which then segregate the protein asymmetrically to produce a hair and a socket cell. d-Numb is similarly used by a variety of neural and nonneural precursor cells to generate cellular diversity during development (reviewed in Betschinger and Knoblich, 2004; Prokopendo and Chia, 2005).

Mammalian Numb homologs are encoded by two genes, *Numb (m-numb*) and *numblike* (*Numbl* or *nbl*; Salcini et al., 1997; Zhong et al., 1996, 1997). The importance of Numb-mediated asymmetric cell division in mammalian development is most clearly demonstrated in the embryonic nervous system, where such divisions are used by progenitor cells to balance the competing needs of self-renewal and neuron production during the extended period of neurogenesis. Direct imaging experiments show that mammalian neural progenitor cells behave like stem cells and can divide asymmetrically to generate a daughter progenitor cell (self-renewal) and a neuron (Chenn and McConnell, 1995; Noctor et al., 2004; Qian et al., 1998). The m-Numb protein localizes asymmetrically in neural progenitor cells and can be segregated to only one daughter cell (Cayouette and Raff, 2003; Shen et al., 2002; Zhong et al., 1996, 1997). Loss-of-function studies using mice show that *m-numb* and *Numbl* are

redundant during neurogenesis, but inactivation of both genes causes progenitor cells to deplete prematurely throughout the embryonic nervous system due to an initial overproduction of neurons at the expense of progenitor cells (Petersen et al., 2002, 2004). These findings point to a role for mammalian Numb proteins in enabling neural progenitor cells to balance self-renewal and neuron production by segregating asymmetrically to the progenitor daughter cells to specify their fates. However, m-Numb and Numbl proteins quickly appear in the neuronal daughter cells (Zhong et al., 1997), and their presence is essential for neuronal differentiation (Huang et al., 2005; Petersen et al., 2002). Thus, Numb signaling seems to play two contradictory roles: namely, it promotes progenitor over neuronal fates but is also required for neuronal differentiation. In other words, the ability of mammalian Numb homologs to specify cell fates also appears to be limited to during and/or shortly after mitosis.

A major contributor to this coordinated process of cell-fate determination and cell-cycle progression is the cellular machinery that asymmetrically localizes Numb proteins only during mitosis (reviewed in Betschinger and Knoblich, 2004; Prokopendo and Chia, 2005). Asymmetric localization, however, is not necessary for Numb to specify cell fates; Numb proteins that distribute uniformly in the cytosol are fully functional, although they force the same fates upon the two daughter cells (Knoblich et al., 1997; Zhong et al., 1997). Thus, the asymmetric localization machinery can explain how the two daughter cells become different but not why Numb proteins lose the ability to specify fates after division, raising the possibility that Numb signaling itself is also regulated during cell cycle. The components of Numb signaling, however, remain largely unknown, particularly in mammals. *Drosophila* studies show that Numb acts by inhibiting the transmembrane receptor Notch (Guo et al., 1996; Spana and Doe, 1996), which mediates cell-cell communication in many contexts during metazoan development (reviewed in Artavanis-Tsakonas, 1999). Because Numb also specifies many unrelated fates, it has been postulated that asymmetric Numb presence simply enables the daughter cells to choose differently between two fate options by biasing Notch-mediated cell-cell communication (reviewed in Zhong, 2003). Whether and how this antagonistic Numb-Notch relationship is conserved in mammals, however, remain unclear (Petersen et al., 2006).

In mammalian cells, cell-cycle progression is accompanied by dramatic changes in the morphology of the Golgi apparatus (reviewed in Altan-Bonnet et al., 2004; Colanzi et al., 2003; Shorter and Warren, 2002). During interphase, mammalian Golgi membranes are organized into interconnected stacks of flattened cisternae and confined to the region surrounding the centrioles. When cells enter mitosis, the pericentriolar Golgi stacks undergo extensive fragmentation, and the Golgi fragments are dispersed throughout the cytosol. In this study, we identify a novel mammalian Numb-binding protein, ACBD3, and show that it is an essential Numb partner in cell-fate specification. We also show that the process of Golgi fragmentation and reconstitution regulates the subcellular distribution of ACBD3 and likely represents a novel mechanism for differentially activating intracellular signaling at different phases of the cell cycle.

RESULTS

ACBD3 Is a Novel Numb-Binding Protein

To elucidate the Numb-signaling pathway, we used yeast two-hybrid screen to identify mouse proteins that physically interact with m-Numb and identified a clone (MERRY MAGPIE) encoding an evolutionarily conserved protein (Figure 1A). This protein contains a putative *a*cyl-*c*oenzyme A-*b*inding *d*omain and was later named ACBD3 by the genome project. It was also identified as GCP60 (Sohda et al., 2001) and PAP7 (Li et al., 2001) due to its ability to bind Giantin, a Golgi membrane protein, and a peripheral-type benzodiazepine receptor, respectively.

Deletion analysis revealed that the N-terminal 23 amino acids of m-Numb contained the ACDB3-binding (AB) domain; other m-Numb regions, including the phosphotyrosine-binding (PTB) domain, were incapable of interacting with ACBD3 (Figures 1B and 1D). The AB domain is highly conserved between mouse and d-Numb proteins (Figure 1B), and ACBD3 could also bind to Numbl and d-Numb (Figure 1C). As expected, a fusion protein of Glutathione-S-Transferase (GST) and the C-terminal 366 amino acids of ACBD3 (ACBD3-C), but not the ACB domain (ACBD3-N), could copurify m-Numb from mouse brain extracts (Figure 1E). We also generated ACBD3 antibodies (Figure 2B) and performed coimmunoprecipitation experiments to examine whether ACBD3 and m-Numb interact in vivo. Indeed, in extracts from HeLa cells cotransfected with plasmids expressing m-Numb and a myristoylated form of ACBD3 ($ACBD3^{Myr}$; see Figure 5A below), an m-Numb antibody, but not an unrelated CSN1 antibody (Yang et al., 2002), was able to coprecipitate both the endogenous ACBD3 and the exogenous $ACBD3^{Myr}$ (Figure 1F).

The ACDB3-Interacting Domain Is Essential for Numb Activity

Interestingly, whereas the N-terminal PTB domain (Figure 1B) is essential for Numb activity (Knoblich et al., 1997; Yaich et al., 1998), the first 23 amino acids comprising the AB domain are not part of the PTB (Li et al., 1998). Further deletion analysis revealed that neither the first 7 amino acids of the AB domain nor the last 16 could mediate binding to ACBD3 by themselves. Surprisingly, although removing all 23 amino acids rendered the rest of the m-Numb protein incapable of binding to ACBD3 in yeast two-hybrid assays, removing only part of the AB domain did not abolish binding (data not shown).

To determine whether an intact AB domain is important for m-Numb to specify cell fates, we deleted amino acids 7–23 from m-Numb and expressed the resultant mutant protein ($m\text{-}Numb^{\Delta 7-23}$) in the *Drosophila* SOP lineage,

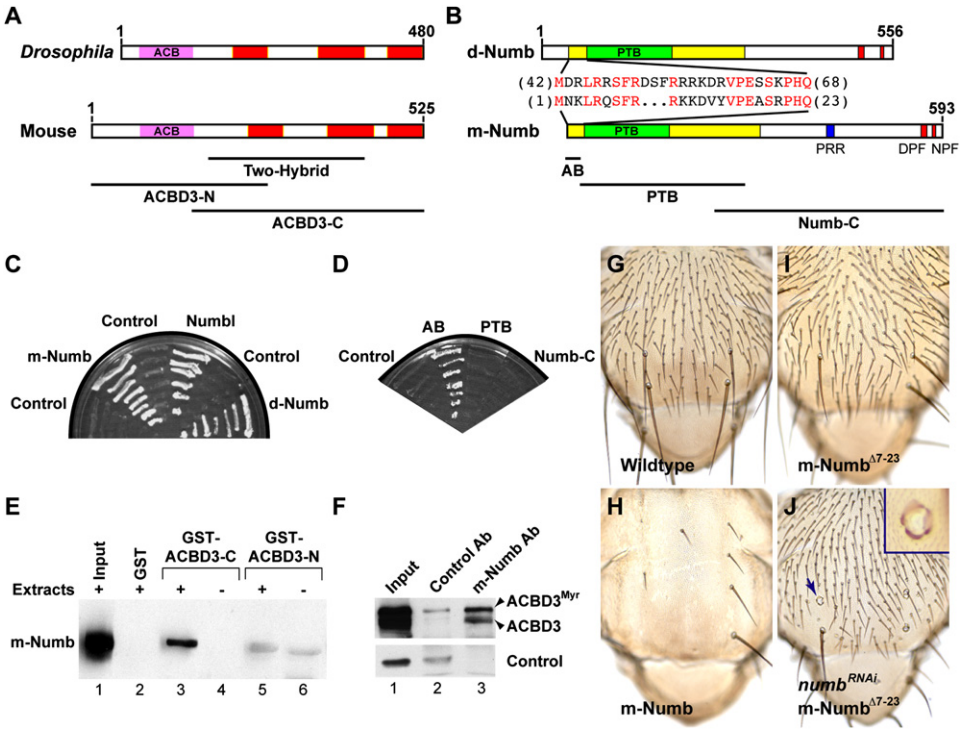

Figure 1. ACBD3 Binds to Numb Proteins through a Functionally Essential Numb Domain

(A) Schematic diagrams of the mouse and *Drosophila* ACBD3 protein. Red boxes indicate over 70% identity in amino acids, and pink boxes indicate ACB domain.

(B) Schematic diagrams of the mouse and *Drosophila* Numb protein. Amino acids are from the respective AB domains. PRR indicates proline-rich region; red boxes indicate DPF or NPF motifs; and yellow boxes (including the PTB domain) indicate over 63% identity in amino acids.

(C) Yeast two-hybrid assays showing the ability of m-Numb, Numbl, and d-Numb to interact with ACBD3 (the two-hybrid fragment in A) or a control vector (without ACBD3).

(D) Yeast two-hybrid assays showing the interaction between different m-Numb regions (the fragments in B) and ACBD3. Control is Numb AB without ACBD3.

(E) Immunoblots using an anti-m-Numb antibody to show the ability of GST- and GST-ACBD3-fusion proteins (the fragments in A) to copurify m-Numb. Presence (+) and absence (−) of mouse brain extracts are as indicated. Input is 10% the amount used in copurification assays.

(F) Immunoblots showing coprecipitation of ACBD3 proteins by an anti-m-Numb antibody. Extracts were from HeLa cells co-transfected with plasmids expressing m-Numb and ACBD3^{Myr}. ACBD3 (endogenous) and ACBD3^{Myr} were identified using the anti-ACBD3-C antibody. An unrelated CSN1 antibody was used to coprecipitate CSN8 as a control. Ab indicates antibody.

(G–J) The notum of a wild-type *Drosophila* and those expressing m-Numb or m-Numb$^{\Delta 7\text{-}23}$ (with or without *d-numb* RNAi). The inset in (J) shows a four-socket mutant ES organ (arrow).

which depends on Numb activity to generate the external sensory (ES) organs on the notum (Rhyu et al., 1994). Overexpression of wild-type m-Numb or d-Numb in SOP cells, which forces the proteins to segregate into both daughter cells, causes balding of the notum (Figure 1H; Rhyu et al., 1994; Tang et al., 2005). This phenotype is due to a transformation of the IIA daughter cell, which normally inherits little Numb, into a second IIB cell (Figure 7L). By contrast, m-Numb$^{\Delta 7-23}$ overexpression, even from multiple copies of the transgene, caused no such defects, with only occasional bristle loss on the notum (Figure 1I). Similarly, whereas m-Numb can suppress the multisocket phenotype caused by *d-numb* double-stranded RNA (dsRNA) that induces RNA interference (RNAi) in the SOP lineage (Tang et al., 2005), m-Numb$^{\Delta 7-23}$ was unable to do so (Figure 1J). The number of ES organs exhibiting the multisocket morphology, which is due to a transformation of the IIB cell into another IIA and of the hair cell into another socket in the absence of d-Numb (Figure 7L), was 30.2 ± 4.4 per notum in *numb*^{RNAi} flies and 25.5 ± 5.4 per notum in those also expressing m-Numb$^{\Delta 7-23}$ (n = 5 each). These findings show that the AB domain is essential for m-Numb to specify cell fates. We point out that m-Numb$^{\Delta 7-23}$ also lacked the ability to associate with the cell membrane and to localize asymmetrically in neural precursor cells (data not shown).

ACBD3 Mutation Causes Embryonic Lethality in Mice

m-numb and *Numbl* double-mutant mice exhibit widespread defects in embryogenesis and die shortly after embryonic day (E) 9.5 (Petersen et al., 2004, 2006). To determine whether ACBD3 loss causes similar phenotypes,

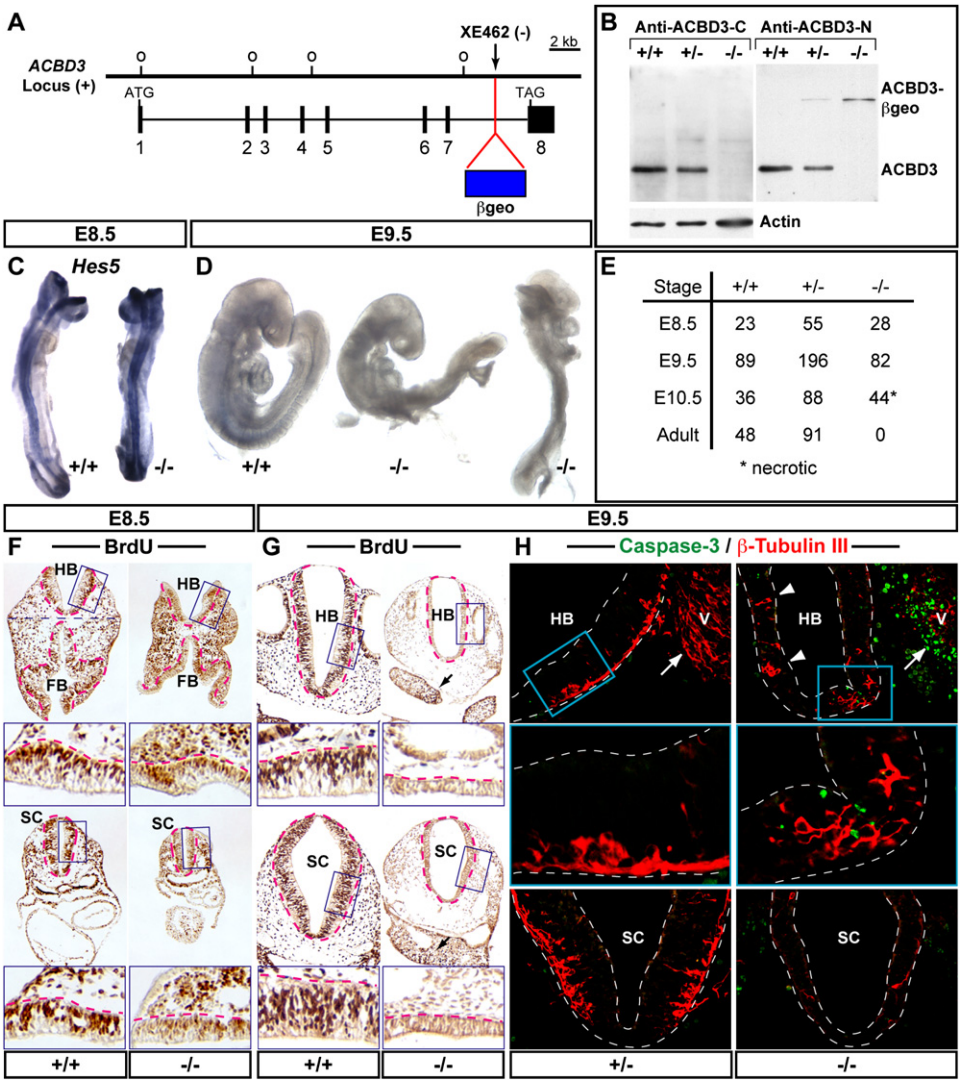

Figure 2. *ACBD3* Is Essential for Embryogenesis and Progenitor Cell Maintenance in Mice

(A) Schematic drawing of the wild-type *ACBD3* locus (+) and XE462 mutant allele (−). Arrow indicates the insertion site of the gene-trap vector (βgeo); O indicates XhoI sites, and 1–8 indicate *ACBD3* exons.

(B) Immunoblots using extracts from E9.5 wild-type (+/+) and XE462 mutant (−/−) embryos to show the specificity of ACBD3 antibodies. Actin was used as a loading control.

(C) *Hes5* expression in E8.5 littermates.

(D) The overall appearance of E9.5 littermates.

(E) Genotypes of the offspring derived from matings between XE462 heterozygous mice.

(F and G) BrdU incorporation (30 min pulse) in E8.5 (F) and E9.5 (G) littermates. Red dashed lines outline the neural tube, and arrows in (G) point to BrdU-labeled cells outside of the nervous system. Boxed areas are magnified below each panel. The top left panel in (F) is a composite of two sections (blue dotted line). FB indicates forebrain; HB indicates hindbrain; and SC indicates spinal cord.

(H) E9.5 littermates double labeled with antibodies against cleaved Caspase 3 (in green) and β-Tubulin III (in red). Boxed areas are magnified below each panel. V indicates trigeminal ganglion.

we identified two lines of embryonic stem (ES) cells (XE462 and XC653) in which a copy of the *ACBD3* gene was mutated due to a gene-trap insertion (Skarnes et al., 1995). Only the XE462 cells yielded mice carrying the mutation, and we mapped the insertion site to near the middle of the last intron of *ACBD3* (Figure 2A). As expected, the wild-type ACBD3 protein was absent in extracts from XE462 homozygous (−/−) embryos, as revealed by ACBD3 antibodies (Figure 2B). Because the gene encoding a β-Galactosidase (β-Gal) and Neomycin phosphotransferase fusion protein (βgeo) in the gene-trap vector contains a splicing acceptance site (Skarnes et al., 1995), the XE462 allele produced an ACBD3-βgeo fusion protein lacking the C-terminal 70 amino acids encoded

by the last *ACBD3* exon. The larger ACBD3-βgeo protein could be identified using an antibody against the N-terminal region of ACBD3 (Figure 2B, right) or a β-Gal antibody (Figure S2).

XE462 heterozygous mice were viable, fertile, and indistinguishable from their wild-type littermates. Matings between XE462 heterozygous mice did not yield homozygous mutants postnatally, indicating that *ACBD3* is essential for embryogenesis (Figure 2E). At E8.5, XE462 homozygous embryos were a bit smaller in size but morphologically indistinguishable from their wild-type littermates (Figure 2C). By E10.5, however, the mutant embryos became necrotic and were at various stages of being absorbed. E9.5 XE462 mutants were about one-half to two-thirds the size of the wild-type littermates (Figure 2D), and many were defective in axial turning and neural tube closure (Figure 2D, the embryo on the right). The phenotype exhibited by *ACBD3* mutants was stronger than that seen in *m-numb* or *Numbl* single mutants (Petersen et al., 2004; Zhong et al., 2000) and more similar to that exhibited by *m-numb* and *Numbl* double mutants. The *ACBD3* phenotype, however, was more variable and in general less severe.

ACBD3 Mutant Embryos Exhibit Defects in Progenitor Cell Maintenance

Loss of both *m-numb* and *Numbl* causes severe thinning of the neuroepithelium due to premature progenitor cell depletion (Petersen et al., 2002, 2004). To examine whether *ACBD3* mutation causes similar defects, we analyzed E9.5 mutants that were morphologically similar to the wild-type (Figure 2D, the middle embryo). Indeed, sectioning revealed that the thickness of the neuroepithelium in most *ACBD3* mutants (16/18) was less than half of that in their wild-type or heterozygous littermates throughout the developing nervous system (Figures S1 and 2G). Since the vast majority of cells in the wild-type neuroepithelium at E9.5 are progenitor cells, severe thinning of the neuroepithelium in *ACBD3* mutants indicates dramatic reductions in progenitor cell numbers. We also analyzed *ACBD3* mutants at E8.5, before the onset of neurogenesis. Although the E8.5 mutant neuroepithelium was a bit thinner than that in the wild-type, the mutant neuroepithelial cells expressed *Hes5* (Figure 2C), a neural progenitor cell marker (Petersen et al., 2004), and were able to incorporate bromodeoxyuridine (BrdU; Figure 2F; 30 min pulse). BrdU is taken up by proliferating cells during DNA synthesis (S phase). These findings show that neural progenitor cells in *ACBD3* mutants are specified and initially capable of proliferation. Thus, the observed thinning of the E9.5 mutant neuroepithelium is likely caused by defects in progenitor cell maintenance.

An antibody against cleaved Caspase-3 revealed no marked increases in apoptosis in the mutant neuroepithelium (Figure 2H, in green, between the dashed lines), and similar results were obtained using TUNEL staining (data not shown). On the other hand, BrdU labeling (30 min pulse) revealed that many E9.5 *ACBD3* mutants (9/18) contained only a few scattered S phase cells in the neuroepithelium (Figure 2G, dashed lines) even though many such cells were present outside of the nervous system (Figure 2G, arrows). Mitotic cells were also fewer in numbers but could be observed in the mutant neuroepithelium using markers like phospho-Histone H3 (P-H3) and Ki-67 (data not shown). These findings suggest that the failure to maintain neural progenitor cells in *ACBD3* mutants is not caused by cell death. It is unclear, however, whether the mutant cells are defective in cell-cycle progression or exit cell cycle to become neurons but fail to differentiate. Staining using β-Tubulin III did not reveal increases in neuron numbers in *ACBD3* mutants, but neurons could be observed spanning the entire width of the mutant neuroepithelium in some regions (Figure 2H, boxed areas and arrowheads). Interestingly, E9.5 *ACBD3* mutants exhibited dramatic increases in cell death in the trigeminal ganglia (Figure 2H, arrows), a region of the peripheral nervous system normally containing large numbers of neurons. *m-numb* and *Numbl* loss also cause neuronal death (Petersen et al., 2002).

We emphasize that the XE462 allele, which produces a truncated ACBD3 protein (Figure 2B), is likely not a gain-of-function or dominant-negative mutation since heterozygous mice do not exhibit defects. It is also unlikely that the mutant ACBD3 protein causes phenotypes by preventing Numb from binding to other essential partners to indirectly affect Numb activity since reducing m-Numb expression by 90% does not affect mouse neurogenesis even in the absence of Numbl (Petersen et al., 2006). Both m-Numb and Numbl proteins were present in *ACBD3* mutant embryos, and, conversely, their loss also did not affect the expression and subcellular distribution of ACBD3 (Figure S3).

ACBD3 Is Ubiquitously Expressed and Associates with the Golgi Apparatus

m-numb and *Numbl* are widely expressed during embryogenesis (Petersen et al., 2004; Zhong et al., 1997). To determine if this is also true for *ACBD3*, we first examined β-Gal activity in XE462 heterozygous embryos, which would be expected to produce βgeo from the *ACBD3* promoter. Indeed, β-Gal activity could be detected in most, if not all, cells in E7.5 and E10.5 embryos (Figure 3A). We further confirmed this ubiquitous pattern of expression using the ACBD3-N antibody; ubiquitous ACBD3 immunoreactivity could be observed in all embryos that we examined between E8.5 and E15.5 (Figures 3B–3I and data not shown).

To determine whether Numb and ACBD3 proteins colocalize within the cells, we analyzed ACBD3 expression in more detail in neuroepithelial cells. Interestingly, although the two proteins were expressed in virtually all neurons and progenitor cells within the embryonic nervous system, they had distinct patterns of subcellular distribution. Whereas m-Numb and Numbl are in the cytosol and largely associate with the cell membrane in neural progenitor cells (Figure 3H, in red; Zhong et al., 1996,

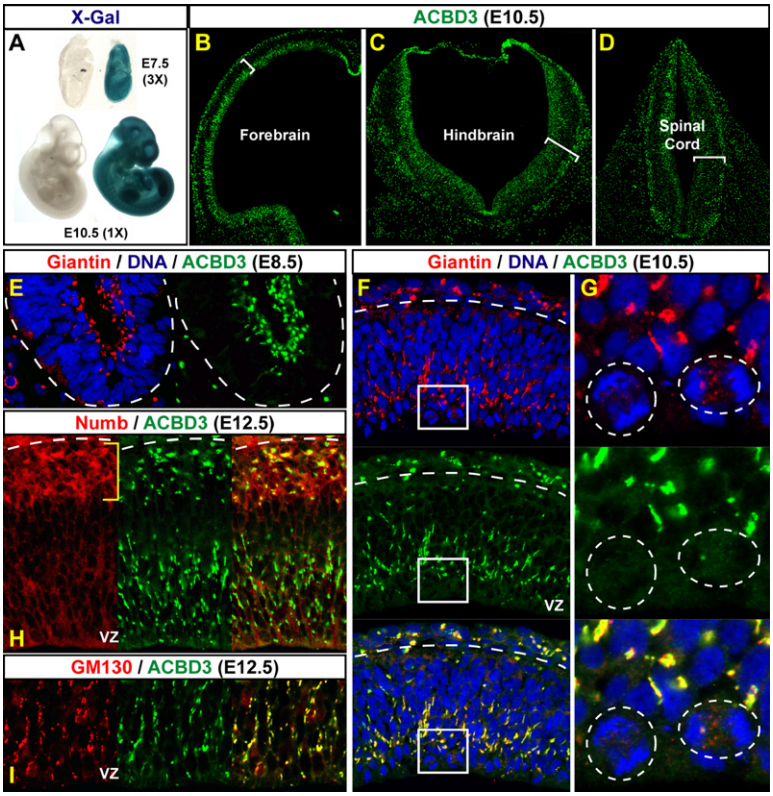

Figure 3. ACBD3 Expression during Mouse Embryogenesis

(A) X-gal staining of wild-type (left) and XE462 heterozygous (right) embryos. E7.5 embryos are shown at a higher magnification (~3×) than the E10.5 embryos.

(B–D) ACBD3 immunoreactivity in a wild-type E10.5 embryo. Brackets indicate the width of the neuroepithelium. Images are shown at a lower magnification than those in (E)–(I).

(E–G) Sections from E8.5 (E; hindbrain) and E10.5 (F and G; forebrain) wild-type embryos double labeled with antibodies against ACBD3 (in green) and Giantin (in red). Propidium iodide was used to stain DNA (in blue). Boxed areas in (F) are magnified in (G). Dashed lines outline the neuroepithelium, and dashed circles outline dividing cells.

(H) A section from an E12.5 wild-type embryo (forebrain) double labeled with antibodies against ACBD3 (in green) and Numbl (in red). Bracket indicates cortical plate and VZ indicates ventricular surface.

(I) A section from an E12.5 wild-type embryo (forebrain) double labeled with antibodies against ACBD3 (in green) and GM130 (in red). Only the ventricular zone is shown.

1997), ACBD3 was concentrated into dot- or string-like morphologies (Figures 3E–3I, in green). In cultured cells, ACBD3 has been shown to associate with the Golgi by localizing to the cytoplasmic face of the Golgi membranes (Sohda et al., 2001). Since the Golgi morphology in neuroepithelial cells has not been characterized, we performed double-labeling experiments using antibodies against ACBD3 and two components of the Golgi matrix, Giantin and GM130 (Shorter and Warren, 2002; Sohda et al., 2001), to determine if ACBD3 also associates with the Golgi in neural progenitor cells. Indeed, ACBD3 immunoreactivity overlapped nicely with that of the Giantin (Figure 3F) and GM130 (Figure 3I).

In most regions of the embryonic central nervous system, the neuroepithelium during early stages of neurogenesis contains a single layer of progenitor cells that forms the ventricular zone (reviewed in McConnell, 1995). Neural progenitor cells, which have thin processes touching both the ventricular and pial surface, replicate DNA with their nuclei in the outer (basal) half of the ventricular zone, and the nuclei then translocate apically in G_2 phase toward the ventricular surface where cells undergo mitosis. This gives the ventricular zone a multilayered appearance. Newborn neurons migrate through the ventricular zone and settle in the outer neuroepithelium, the mantle zone (or cortical plate in the dorsal forebrain), for terminal differentiation. Immunostaining experiments further revealed that in interphase neural progenitor cells, the Golgi likely exists as a single string-like structure and is located adjacent to the apical side of the nucleus or within the apical fiber. This is most apparent in the E8.5 neuroepithelium, where Golgi staining could be observed in the apical one-third of the neuroepithelium but not the basal two-thirds (Figure 3E). Golgi and ACBD3 signals started to appear in the outer, mantle zone with the arrival of neurons (Figure 3H, bracket), but the signals remained more numerous near the inner part within the ventricular zone (Figures 3F and 3H).

ACBD3 Is Released into the Cytosol after Golgi Fragmentation during Mitosis

Interestingly, whereas ACBD3 staining overlapped nearly completely with that of the Golgi markers in postmitotic neurons in the mantle zone and in interphase progenitor cells within the ventricular zone, the two signals became distinct in dividing (M phase) cells near the ventricular surface. In such cells (Figure 3G, dashed circles), whereas the Giantin antibody stained large numbers of scattered vesicles, ACBD3 staining was much weaker and more smoothly cytosolic. In dividing cells, ACBD3 also did not colocalize with GM130, which exhibited a haze-like pattern of distribution (data not shown). Giantin and GM130 distribution actually reflects what is thought to be a unique property of mammalian cells in culture; namely, they fragment their Golgi right before entering into mitosis (reviewed in Altan-Bonnet et al., 2004; Colanzi et al., 2003; Shorter and Warren, 2002). Neural progenitor cells apparently also do so.

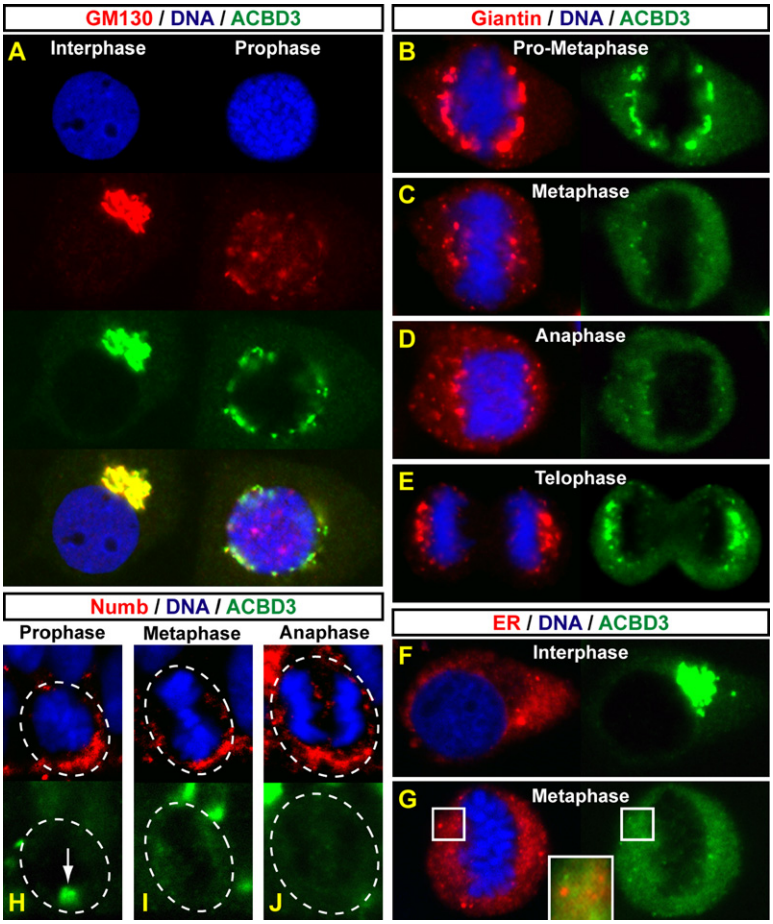

Figure 4. Subcellular Distribution of the ACBD3 Protein during Cell Cycle

(A) NIE115 cells triple labeled for ACBD3 (in green), GM130 (in red), and DNA (in blue).

(B–E) NIE115 cells triple labeled for ACBD3 (in green), Giantin (in red), and DNA (in blue).

(F and G) NIE115 cells triple labeled for ACBD3 (in green), ERp72 (in red), and DNA (in blue). The inset in (G) is the merged image of the boxed areas shown at a higher magnification (ACBD3 and ERp27 staining only).

(H–J) Neural progenitor cells from an E10.5 wild-type embryo (hindbrain section) triple labeled for ACBD3 (in green), Numb (m-Numb and Numbl, in red), and DNA (in blue). Dashed lines outline the cell body. Arrow points to the concentrated ACBD3 staining.

We further examined whether ACBD3 is indeed released into the cytosol during mitosis by using cell lines, where Golgi fragmentation has been extensively studied. In NIE115 cells, a neuroblastoma cell line, the Golgi existed as a prominent structure during interphase (Figure 4A, left). By early prophase, however, it had already fragmented, and different Golgi components exhibited distinct patterns of distribution; whereas GM130 was dispersed into haze-like structures (Figure 4A, right, in red), Giantin was present in large blobs (Figure 4B, in red). ACBD3 largely colocalized with Giantin in prophase and prometaphase cells (Figure 4B), but, by late metaphase and throughout anaphase, ACBD3 distribution had become fairly smoothly cytosolic and was distinct from that of the Giantin (Figures 4C and 4D), GM130 (data not shown), or an ER marker (Figure 4G). ACBD3 started to return to the reforming Golgi during telophase (Figure 4E) and presumably became Golgi-associated again shortly afterwards. Other cell lines showed similar changes in ACBD3 distribution during cell cycle (data not shown).

ACBD3 Release into the Cytosol Occurs after Numb Distribution Becomes Asymmetric

Previous studies show that asymmetric Numb localization in neural progenitor cells first occurs during prophase (Zhong et al., 1996). Since ACBD3 likely does not become mostly cytosolic until metaphase, we performed double-labeling experiments using sections from wild-type mouse embryos to ascertain if ACBD3 release occurs after Numb distribution has become asymmetric during neurogenesis. In these experiments, we used a Numbl antibody that recognizes both m-Numb and Numbl (Zhong et al., 1997). As expected, whereas concentrated ACBD3 staining could still be detected in prophase neural progenitor cells, Numb proteins had already become asymmetrically localized to the apical or apical-lateral cell membrane in the same cells (Figure 4H). By late metaphase, however, ACBD3 staining could be observed fairly uniformly in the cytoplasm along with the asymmetrically localized Numb (Figures 4I and 4J). The ACBD3-βGeo-fusion protein produced by the XE462 allele localized to the Golgi and exhibited changes in subcellular distribution during cell cycle like the wild-type protein (Figure S2).

Continuing Cytosolic Presence of ACBD3 Inhibits Neurogenesis in Mice

That ACBD3 becomes cytosolic only during mitosis raises an interesting possibility that ACBD3 regulates Numb signaling by activating its ability to specify cell fates only when Numb activity is needed to distinguish the two

daughter cells. In both mice and *Drosophila,* Numb proteins associate with the cytoplasmic side of the cell membrane in asymmetrically dividing precursor cells (Rhyu et al., 1994; Zhong et al., 1996). We therefore added a myristoylation site to the N terminus of ACBD3 to examine whether forcing ACBD3 to remain in the cytosol throughout the progenitor cell cycle affects neurogenesis. Myristoylation is a lipid modification that anchors a protein to the cytoplasmic side of the cell membrane. As expected, the resultant protein, ACBD3^{Myr}, could be detected outlining the cell contour in the cytosol in interphase cells (Figure 5A, arrowheads), although some remained associated with the Golgi (Figure 5A, arrow).

To examine the effect of a continuing cytosolic presence of ACBD3 during progenitor cell divisions, we used a Cre-*loxP*-based strategy to conditionally express ACBD3^{Myr} in mice. We generated transgenic mice *(Flox-STOP-ACBD3^{Myr})* carrying a hybrid gene in which a cDNA encoding ACBD3^{Myr}, tagged with a MYC epitope, was fused to the 3′ end of a cassette containing transcription-terminating poly-A (STOP) sites flanked by two *loxP* sites (Figure 5B). The hybrid gene was under the control of a chick *β-actin* promoter that is active in all cells but would produce the exogenous protein only after being exposed to Cre (Figure 5C). As expected, mice carrying only the *Flox-STOP-ACBD3^{Myr}* transgene were normal (data not shown).

We used *Nestin-Cre*, which becomes active in neural progenitor cells throughout the embryonic nervous system shortly after E8.5 (Petersen et al., 2002), to activate ACBD3^{Myr} production and analyzed the resultant mutants at E10.5. Misexpression of ACBD3^{Myr} in neuroepithelial cells markedly inhibited neuron production throughout the developing nervous system, particularly in the ventral spinal cord and hindbrain, where neurogenesis is more advanced at E10.5 (Figures 5D–5L, in red). Moreover, this reduction in neuron production was not caused by defects in the number nor proliferation of neural progenitor cells (Figure 6Z). In ACBD3^{Myr} mutants, the thickness of the ventricular zone, where progenitor cells reside, was not markedly different from that of the wild-type embryos. The mutant ventricular zone cells expressed progenitor cell markers like *Hes5* (Figures 5M–5R) and the M phase marker P-H3 (Figures 5D–5H, in blue). Neural progenitor cells in ACBD3^{Myr} mutants were also fully capable of incorporating BrdU (Figures 6F–6H, in green). The mutant neuroepithelium was thinner due to reductions in neuron production, and the ventricular zone actually occupied nearly its entire thickness.

The phenotype exhibited by embryos misexpressing ACBD3^{Myr} is remarkably similar to that observed when neural progenitor cells are forced to misexpress Numbl^{SYM}, a symmetrically segregating splicing variant of mouse Numbl (Zhong et al., 1997). In a study to be described elsewhere, we show that symmetric Numbl^{SYM} segregation inhibits neuron production by forcing both progenitor daughter cells to choose self-renewal over differentiation, which in turn triggers a homeostasis mechanism that maintains progenitor cell numbers at wild-type levels by eliminating overproduced cells through apoptosis (K.Z. and W.Z., unpublished data). ACBD3^{Myr} misexpression appeared to trigger the same homeostatic response; in the mutant neuroepithelium, double-labeling experiments using the neuronal marker MAP2 and TUNEL staining (Figures 5D–5H) or an antibody against cleaved Caspase-3 (Figures 5I–5L) revealed dramatic increases in cell death within the ventricular zone but not among neurons of the mantle zone. We, however, cannot exclude the possibility of neuronal death due to a lack of markers for young neurons. ACBD3^{Myr} presence in XE462 heterozygous embryos also caused reductions in neuron production and increases in cell death within the ventricular zone (Figure S4), suggesting that the defects are due to a continuing cytosolic presence of ACBD3 and not its overexpression at the Golgi.

Cytosolic ACBD3 Can Act Together with Numb during Mouse Neurogenesis

The expression level of a transgene in mice is influenced by its chromosome location. Not surprisingly, the severity of the mutant phenotype varied among the seven independently generated ACBD3^{Myr} lines that we examined (data not shown), which also indicates that ACBD3^{Myr} presence in these mutants likely affected only a fraction of the neural progenitor cells. We therefore used *Nestin-Cre* to coexpress ACBD3^{Myr} and Numbl^{SYM} in the embryonic nervous system to examine whether their copresence could lead to more severe defects. Indeed, compared to those expressing ACBD3^{Myr} (Figures 6F–6H) or Numbl^{SYM} (Figures 6P–6R) alone, ACBD3^{Myr}+Numbl^{SYM} mutants (Figures 6K–6M) contained markedly fewer neurons in the neuroepithelium as revealed by the expression of the neuronal marker Hu (in red). As expected, the mutant neuroepithelium continued to express *Hes5* (data not shown), indicating that the remaining cells were neural progenitor cells. To further ascertain that ACBD3^{Myr} and Numbl^{SYM} comisexpression more significantly inhibited neurogenesis, we stained the neuroepithelium with an antibody against Islet1, a nuclear transcription factor, to reveal the number of motor neurons in the ventral spinal cord (Figures 6N and 6O). Quantitative analysis confirmed that ACBD3^{Myr}+Numbl^{SYM} mutants had significantly fewer Islet1-positive cells in the ventral spinal cord than those expressing ACBD3^{Myr} or Numbl^{SYM} alone (Figure 6Z, right panel).

Interestingly, ACBD3^{Myr} and Numbl^{SYM} coexpression also reduced the number of S phase cells labeled by a 30 min pulse of BrdU in the neuroepithelium (Figure 6Z, left panel), which may indicate a slowdown of cell-cycle progression. The ventricular zone in the neuroepithelium of ACBD3^{Myr}+Numbl^{SYM} mutants was also thinner (Figures 6K–6M) than that in the wild-type. This, however, may not indicate that the mutants had fewer progenitor cells; an absence of migrating newborn neurons could also make the ventricular zone appear thinner.

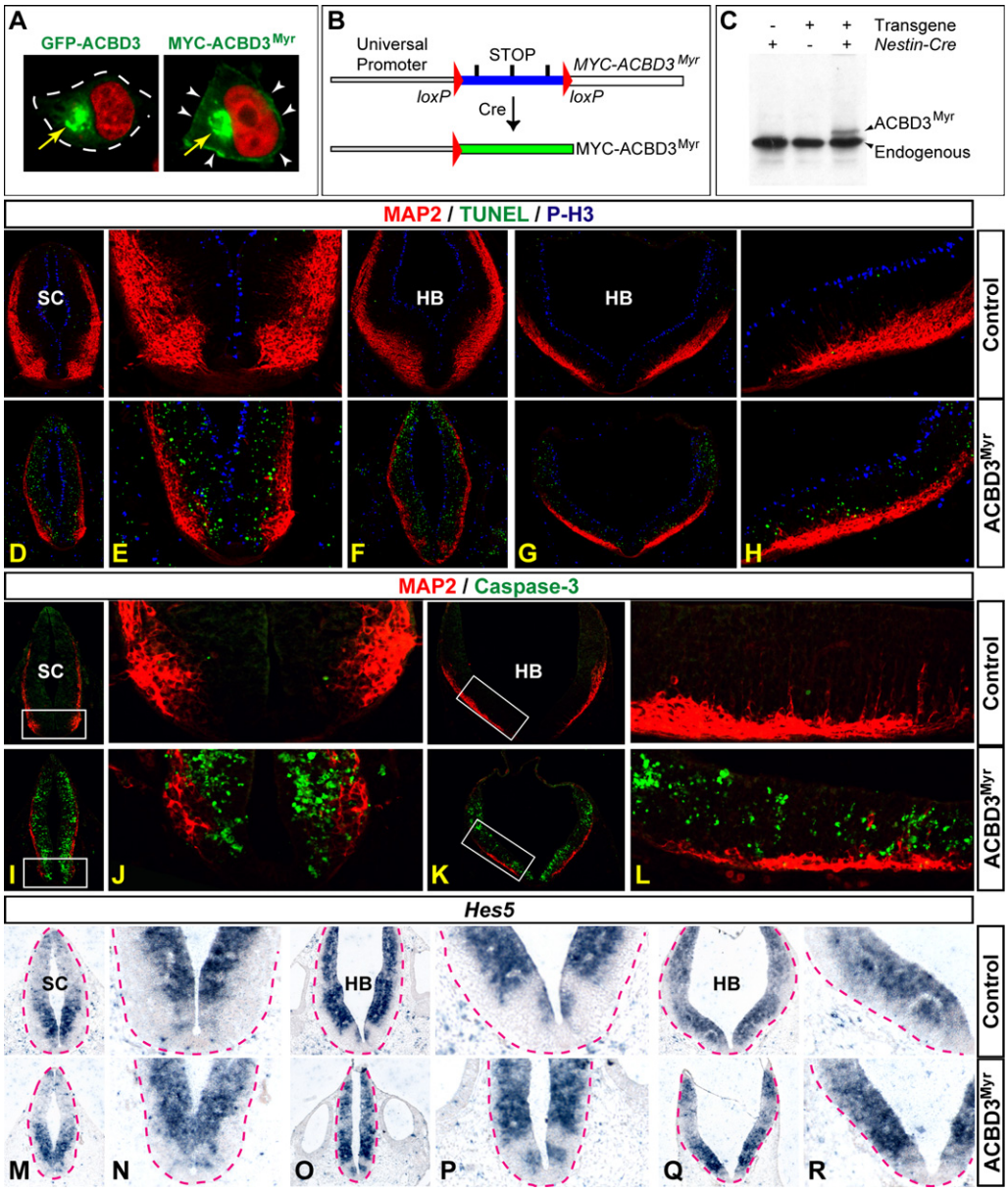

Figure 5. Continuing ACBD3 Presence in the Cytosol during the Progenitor Cell Cycle Inhibits Neuron Production during Mouse Neurogenesis

(A) The distribution of wild-type ACBD3 (GFP-tagged, left) and ACBD3^{Myr} (MYC-tagged, right) in interphase HeLa cells. Arrows point to the Golgi, whereas arrowheads and dashed line indicate the cell membrane.

(B) Schematic drawings of the strategy used to conditionally express ACBD3^{Myr} in mice. Not shown is a 1.3 kb intron, with splicing donor and acceptance sites, between the promoter and the first *loxP* site. Universal promoter indicates chick β-*actin* promoter and STOP indicates three poly-A sites.

(C) An immunoblot using anti-ACBD3-C antibody to show ACBD3^{Myr} presence only in mice carrying both *Flox-STOP-ACBD3^{Myr}* and *Nestin-Cre*. Protein extracts were from E10.5 embryos.

(D–H) Sections from E10.5 wild-type control and ACBD3^{Myr} embryos triple labeled for neurons (MAP2), cell death (TUNEL), and M phase cells (P-H3). (E) and (H) are respective high-magnification images of part of (D) and (G). HB indicates hindbrain and SC indicates spinal cord.

(I–L) Sections from E10.5 wild-type control and ACBD3^{Myr} embryos double labeled for MAP2 and cleaved Caspase-3. (J) and (L) are the respective boxed areas in (I) and (K) in higher magnification.

(M–R) *Hes5* expression in E10.5 wild-type and ACBD3^{Myr} embryos. (N), (P), and (R) are respective high-magnification images of part of (M), (O), and (R). The hindbrain regions shown in (F) and (O) are at the otic vesicle level and caudal to those shown in (G), (K), and (Q).

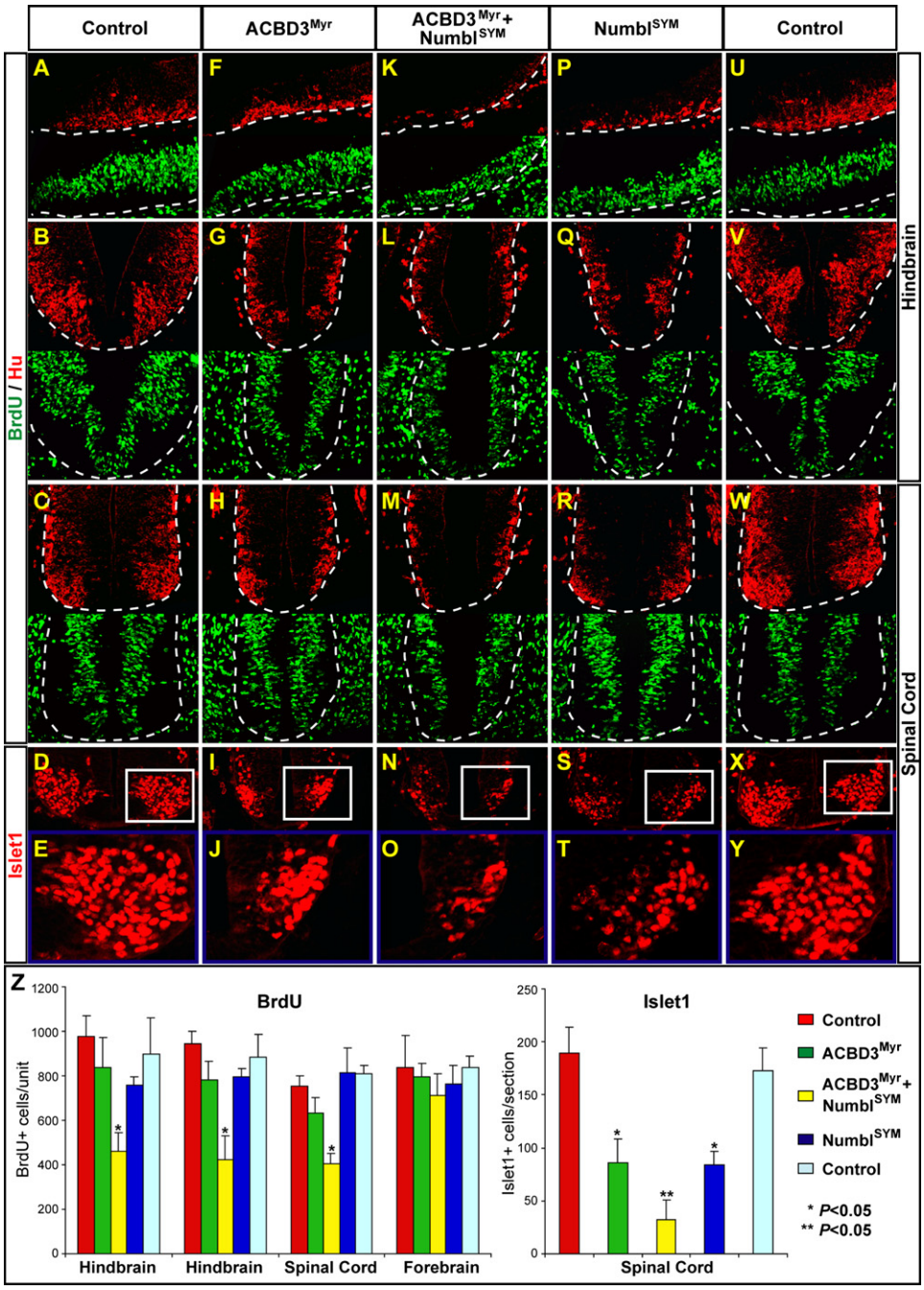

Figure 6. ACBD3 and Numb Proteins Can Act Together during Mouse Neurogenesis

(A–Y) E10.5 wild-type control embryos and those misexpressing ACBD3^{Myr} and Numbl^{SYM} alone or together. Sections were doubled labeled for BrdU (30 min pulse) and Hu or stained for Islet1. (A)–(O) and (P)–(Y) are littermates, respectively. Boxed areas are magnified below each panel. Dashed lines outline the neuroepithelium.

(Z) Quantitative analysis of BrdU-labeled (left) and Islet1-positive (right) cells in the neuroepithelium. Error bars indicate standard deviation, and statistic significance (p value) is based on Student's t test by comparing the respective mutants to wild-type embryos (*) or to ACBD3^{Myr} and Numbl^{SYM} single mutants (**).

Cytosolic ACBD3 Depends on and Acts Synergistically with Numb to Specify Cell Fates

While the findings described above support the notion that ACBD3 and Numb proteins are partners in cell-fate specification, it is unclear whether they act in the same signaling pathway or in two parallel pathways. To distinguish these possibilities, we introduced ACBD3^{Myr} into *Drosophila* SOP cells. ACBD3 can interact with d-Numb

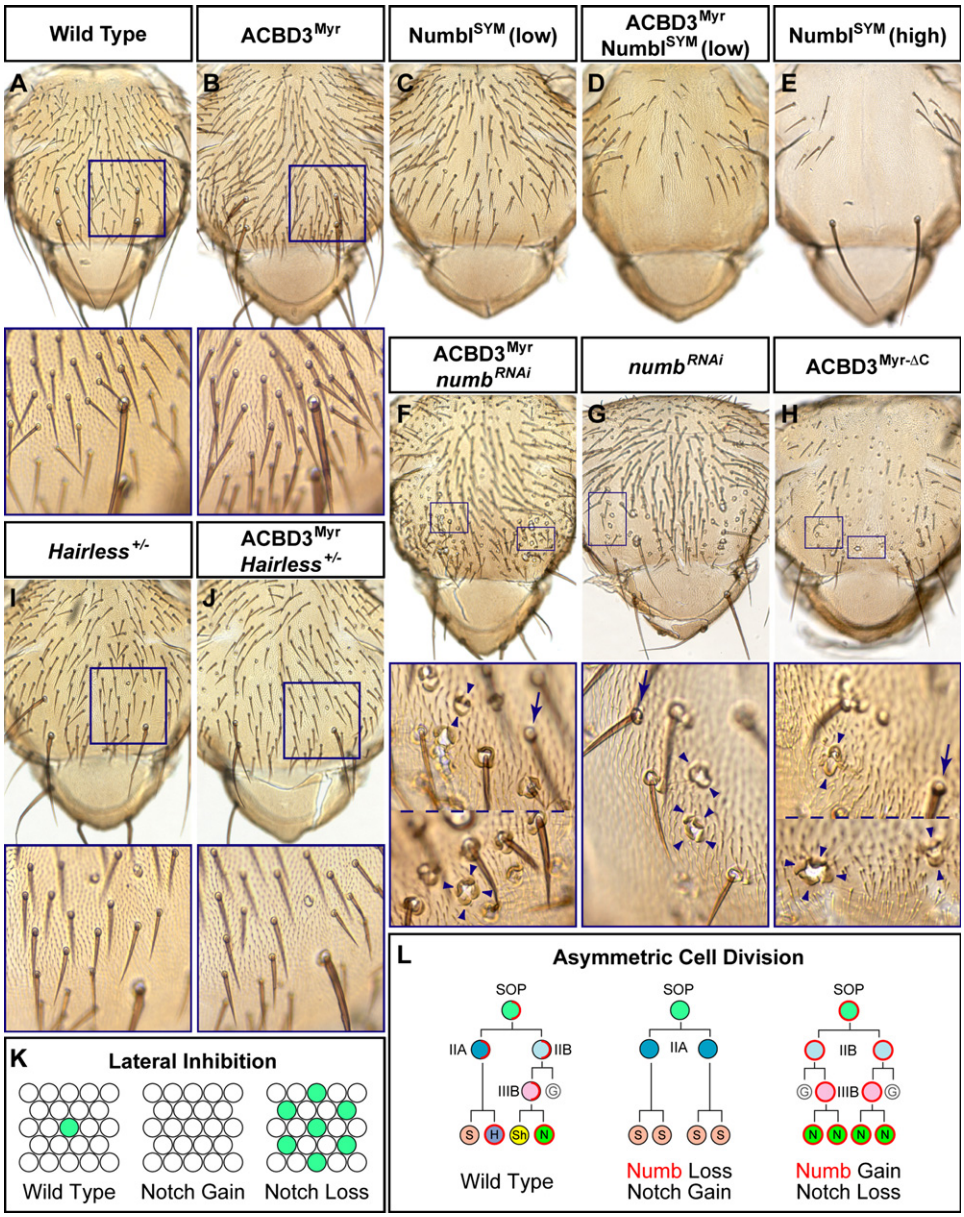

Figure 7. Cytosolic ACBD3 Can Inhibit Notch Signaling and Act Synergistically with Numb to Specify Cell Fates in *Drosophila*

(A–E) The notum of a wild-type *Drosophila* and those expressing ACBD3^{Myr} and Numbl^{SYM} alone or together. Numbl^{SYM} expression is higher in (E) than (C) and (D). Boxed areas are magnified below each panel. Magnification of the boxed areas is lower in (A), (B), (I), and (J) (2.5×) than (F)–(G) (5×).

(F–H) The notum of *Drosophila* expressing *d-numb* dsRNA together with ACBD3^{Myr} (F), dsRNA alone (G), or ACBD3$^{Myr\text{-}\Delta C}$ (H). Arrows point to a wild-type ES organ, whereas arrowheads point to mutant ES organs with two or four sockets.

(I and J) The notum of *H* heterozygous *Drosophila* with (J) or without (I) ACBD3^{Myr} presence.

(K) Schematic drawings of the effect of changes in Notch activity on lateral inhibition. Green circles indicate SOP cells and white circles indicate ectodermal cells competent to become SOP cells.

(L) Schematic drawings of the effect of changes in Notch and Numb activity on asymmetric cell division within the SOP lineage. S indicates socket cell; G indicates glial cell; H indicates hair cell; Sh indicates sheath cell; and N indicates neuron.

(Figure 1C), and *Drosophila* has a highly conserved ACBD3 homolog (Figure 1A; Sohda et al., 2001). Imaging experiments show that SOP cells segregate Numb in a nearly all-or-nothing fashion (Gho and Schweisguth, 1998; Rhyu et al., 1994). Thus, ACBD3^{Myr} alone would not be expected to affect SOP divisions if its ability to specify cell fates requires Numb presence. Indeed, whereas Numb overexpression causes balding of the notum due to an absence of IIA-derived hair and socket cells (Figures 1H and 7E; Rhyu et al., 1994; Tang et al., 2005),

ACBD3^{Myr} presence did not cause balding but instead produced more hairs, each with an accompanying socket, on the notum (Figures 7B; see discussion below regarding increases in hair numbers).

To examine if ACBD3 can enhance Numb activity, we coexpressed ACBD3^{Myr} and the symmetrically segregating Numbl^{SYM} in the wild-type SOP lineage. At low levels, symmetric Numbl^{SYM} inheritance was insufficient to overcome the asymmetric presence of the endogenous d-Numb protein in many SOP daughter cells and caused only mild balding of the notum (Figure 7C). However, ACBD3^{Myr} and Numbl^{SYM} coexpression resulted in more severe balding of the notum (Figure 7D), which is a phenotype similar to that caused by high levels of Numbl^{SYM} expression (Figure 7E). This functional synergy was observed between multiple independently generated transgenic lines. Thus, ACBD3 and Numb can act synergistically, but the former requires the presence of the latter to specify cell fates. Not surprisingly, unlike m-Numb and Numbl^{SYM} (Tang et al., 2005; data not shown), ACBD3^{Myr} was unable to suppress the multisocket phenotype caused by RNAi-induced d-Numb loss (Figure 7F, arrowheads).

We also expressed a truncated form of ACBD3^{Myr} (ACBD3$^{Myr\text{-}\Delta C}$) in SOP cells to examine whether it could interfere with Numb signaling to cause Numb loss-of-function phenotypes. ACBD3$^{Myr\text{-}\Delta C}$ contains the Numb-binding domain but lacks the C-terminal 154 amino acids that are highly conserved between mouse and *Drosophila* (Figure 1A). ACBD3$^{Myr\text{-}\Delta C}$ presence in the SOP lineage caused decreases in hair numbers (Figure 7H). However, unlike the outcome of forcing SOP cells to symmetrically segregate Numb activity, which causes both hairs and sockets to become absent (Figures 7D and 7E), sockets were present on the notum of ACBD3$^{Myr\text{-}\Delta C}$-misexpression mutants. More importantly, some mutant ES organs exhibited a multisocket morphology (Figure 7H, arrowheads) that is virtually identical to that caused by Numb loss (Figure 7G, arrowheads), further suggesting that ACBD3 can act in the Numb pathway. As expected, hair loss was more severe in flies expressing ACBD3$^{Myr\text{-}\Delta C}$ from multiple copies of the transgene (Figure 7H) than those expressing the mutant protein from one copy (data not shown).

Cytosolic ACBD3 Can Inhibit Notch Signaling

In the *Drosophila* experiments described above, we expressed ACBD3^{Myr} in the SOP cells as well as the ectodermal cells from which they arise (see Experimental Procedures). Interestingly, ACBD3^{Myr} expression also caused more ES organs (hairs with accompanying sockets) to emerge on the notum (Figures 7B). This is reminiscent of classical Notch-signaling mutant phenotypes, which exhibit an overproduction of SOP cells due to a reduction of Notch activity during lateral inhibition, a process that depends on Notch signaling to enable only a single neural precursor to emerge from a group of competent ectodermal cells (Figure 7K; reviewed in Artavanis-Tsakonas, 1999). To verify that ACBD3^{Myr} can inhibit Notch signaling during lateral inhibition, we expressed ACBD3^{Myr} in flies heterozygous for a mutation of *Hairless (H)*, a dominant and specific inhibitor of Notch signaling (Bang et al., 1991). Indeed, increases in Notch signaling due to *H* heterozygosity suppressed the ACBD3^{Myr} phenotype, and the resultant flies exhibited only the *H* mutant phenotype (Figures 7I and 7J). We point out that d-Numb is not involved in lateral inhibition but is expressed during this process (Rhyu et al., 1994). Thus, that ACBD3^{Myr} can inhibit Notch signaling in this context is not inconsistent with the notion that ACBD3 activity requires Numb presence. Future study, however, is necessary to ascertain this. ACBD3^{Myr} associated with the cell-membrane in *Drosophila*, and, for reasons that are unclear, the wild-type mouse ACBD3 protein did not appear to have similar activity in *Drosophila* (data not shown).

DISCUSSION

ACBD3 and Numb Proteins Are Interdependent Partners in Cell-Fate Specification

We have reported here that ACBD3 and Numb proteins can physically interact through a functionally indispensable Numb domain (Figure 1) and that changes in their activity can cause remarkably similar defects in the ability of neural progenitor cells to balance self-renewal and neuron production during mouse neurogenesis (Figures 2, 5, and 6; Petersen et al., 2002, 2004). These findings strongly suggest that ACBD3 is a component of the Numb-signaling pathway in cell-fate specification, at least in mammals.

Neural progenitor cells segregate Numb proteins to one daughter cell but distribute ACBD3 symmetrically during asymmetric divisions (Figure 4). Our findings further suggest that Numb and ACBD3 do not act in two parallel pathways but are codependent partners in the same pathway. When assayed in *Drosophila,* ACBD3^{Myr} lacks the ability to specify cell fates by itself but allows Numbl^{SYM} to transform fates at levels that otherwise show little activity (Figure 7). Numb and ACBD3 likely also do not act in a simple hierarchy since they are not required for each other's expression and proper subcellular distribution (Figure S3). Thus, that Numb and ACBD3 loss can both cause severe defects in progenitor cell maintenance during mouse neurogenesis (Figure 2; Petersen et al., 2002, 2004) indicates that specifying Numb-dependent cell fates likely requires the presence of both proteins.

Why, then, can ACBD3^{Myr} misexpression alone cause neurogenesis defects in mice? One possibility is that asymmetric Numb segregation by mammalian neural progenitor cells is not an all-or-nothing event. For example, progenitor cells that divide vertically may segregate the apically localized Numb proteins to both daughter cells, but in different amounts, to generate two different daughter cells (Figure 4J). This may allow ACBD3^{Myr} to raise Numb activity level above threshold in prospective neuronal daughter cells and force them to also choose progenitor fates. Alternatively, newly synthesized Numb proteins

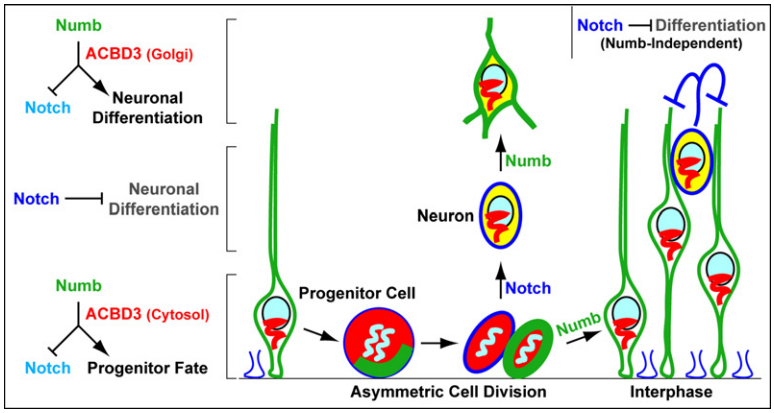

Figure 8. A Model for Differentially Regulating Numb and Notch Signaling during Mammalian Neurogenesis through Golgi Fragmentation and Reconstitution

In this model, Numb proteins (in green) inhibit Notch signaling to specify progenitor cell fates when neural progenitor cells divide asymmetrically to self-renew and produce a neuron, but this occurs only when ACBD3 (in red) is present in the cytosol after Golgi fragmentation during mitosis. Newborn neurons have higher Notch activity, which also prevents them from terminal differentiation. Newly synthesized Numb proteins subsequently accumulate in newborn neurons, but, with ACBD3 localized to the Golgi, they inhibit Notch signaling by tapping into a different pathway to promote neuronal differentiation. Notch activation is also necessary for preventing progenitor cells from differentiating during interphase, and this activity is not affected by Numb presence because ACBD3 associates with the Golgi. Stem cells in other tissues may also use this mechanism to balance self-renewal and differentiation.

may, in the presence of ACBD3^{Myr}, force newborn neurons to become progenitor cells (or die) instead of differentiating.

A Novel Golgi-Based Mechanism for Regulating Intracellular Signaling during Cell Cycle

Our findings also provide evidence that the process of Golgi fragmentation and reconstitution during cell cycle, which has long been recognized as a distinct feature of mammalian cells (reviewed in Colanzi et al., 2003; Shorter and Warren, 2002), regulates Numb activity in cell-fate specification by changing the subcellular distribution of ACBD3 and may represent a novel mechanism for modulating intracellular signaling at different phases of the cell cycle.

Mammalian Numb proteins are expressed by both progenitor cells and neurons and play two distinct roles during neurogenesis: they specify progenitor over neuronal fates but are also required for neuronal survival and differentiation (Huang et al., 2005; Petersen et al., 2002, 2004; Zhong et al., 1997). The dynamic changes in ACBD3 distribution during cell cycle have led us to propose a novel mechanism that can reconcile the two seemingly contradictory roles. In this model (Figure 8), Numb proteins are capable of specifying progenitor fates only during mitosis and/or shortly afterwards, when their partner ACBD3 is also in the cytosol, and ACBD3 sequestration in the Golgi after the division allows newly synthesized Numb proteins to instead promote neuronal differentiation by tapping into a different signaling pathway. This model is supported by the finding that forcing ACBD3 to remain in the cytosol of neural progenitor cells throughout the cell cycle inhibits neuron production (Figures 5). We point out that ACBD3 likely has additional roles in the Golgi, which is involved in posttranslational modification and trafficking of proteins. The Golgi, however, appears to fragment normally in *ACBD3* mutants (Figure S2), suggesting that ACBD3 is not required for this process, which is essential for cell-cycle progression (Sutterlin et al., 2002).

Our findings further raise several interesting possibilities. First, releasing ACBD3 into the cytosol likely is not simply a by-product of Golgi fragmentation but rather a precisely regulated process since ACBD3 initially remains associated with Giantin-positive Golgi blobs after Golgi fragmentation and does not become mostly cytosolic until late metaphase (Figure 4). Second, ACBD3 begins to return to the reconstituting Golgi during telophase (Figure 4E), indicating that commitment to a particular fate may occur during mitosis, when the two daughter cells are being generated. Future study that more directly monitors ACBD3 release and its effect on Numb signaling during cell cycle is necessary to ascertain this. Third, it is conceivable that other proteins may similarly use Golgi association to differentially regulate signaling during cell cycle. Finally, *Drosophila* Golgi exists as dispersed units and does not further fragment during cell cycle (Stanley et al., 1997), raising an interesting question as to whether this Golgi-based mechanism is conserved evolutionarily.

Regulation of Notch Signaling in Mammalian Development: Interphase versus Mitotic Cells

Numb specifies asymmetric cell fates by inhibiting Notch signaling in *Drosophila*, but Numb and Notch mutant mice show similar, not opposite, defects in neurogenesis, particularly progenitor cell maintenance (reviewed in Petersen et al., 2006). Our findings may provide a mechanism to explain these two seemingly contradictory observations.

In *Drosophila,* Notch signaling also enables emerging neural precursor cells to prevent neighboring cells from adopting the same fates (reviewed in Artavanis-Tsakonas,

1999). This process of lateral inhibition, which occurs when cells are at interphase, is not affected by changes in Numb activity, and we have postulated that the Numb-Notch relationship is similarly complex during mammalian neurogenesis (Petersen et al., 2006). In this model (Figure 8), Notch signaling keeps progenitor cells from differentiating during interphase but promotes neuronal fates when cells divide to self-renew and produce a neuron, and Numb proteins do not inhibit Notch activity in interphase progenitor cells but do so during an asymmetric division to specify progenitor fates. Moreover, Notch signaling also inhibits the differentiation of newborn neurons, which occurs only after Notch activity is inhibited by newly synthesized Numb proteins. This model can explain why the neurogenesis phenotypes caused by Numb and Notch loss are similar (but for different reasons). The findings reported here may provide a molecular basis for this complex Numb-Notch relationship—namely, ACBD3 absence in the cytosol not only prevents Numb proteins from inhibiting Notch activity in interphase progenitor cells but also enables them to do so in newborn neurons to promote differentiation by tapping into a different pathway.

How do ACBD3 and Numb act together to inhibit Notch signaling during an asymmetric cell division? It has been postulated that Numb proteins act by directly binding to regulators of endocytosis- and proteasome-mediated degradation to reduce Notch protein level at the cell surface (reviewed in Betschinger and Knoblich, 2004). Interestingly, whereas the domain mediating ACBD3 interaction is essential for Numb function (Figure 1), neither elimination of the binding motifs for endocytic proteins nor simultaneous reduction of proteasome activity affects the ability of Numb proteins to specify cell fates (Tang et al., 2005), raising a distinct possibility that ACBD3 enables Numb proteins to use an endocytosis- and proteasome-independent pathway for cell-fate specification.

ACBD3-Regulated Numb-Notch Signaling: A General Mechanism for Stem Cell Divisions?

Stem cells are involved in tissue generation and maintenance as well as tumorigenesis (Reya et al., 2001). Understanding the mechanisms that balance self-renewal and differentiation is one of the most fundamental issues in stem cell biology. Conceptually, stem cells can balance the two needs as a group by determining the fraction that differentiates and the one that continues to self-renew. This balance can also be achieved by individual stem cells through intrinsically asymmetric divisions. The interplay between ACBD3, Numb, and Notch provides an attractive molecular framework to integrate the two mechanisms. Numb and ACBD3 proteins are widely expressed during embryogenesis (Figure 3; Zhong et al., 1997), and Notch signaling also plays many roles in development (reviewed in Artavanis-Tsakonas, 1999). Therefore, it is tempting to speculate that this Golgi-based mechanism for regulating Numb and Notch signaling is a general scheme utilized by a variety of stem cells in organogenesis and tissue maintenance.

EXPERIMENTAL PROCEDURES

Cloning of Mouse *ACBD3* and Protein-Protein Interaction Assays

m-Numb (amino acids 1–282) was used to isolate a clone of *ACBD3*, encoding amino acids 184–461, from a yeast two-hybrid library (E10.5 mouse embryos; Clontech). The full-length *ACBD3* cDNA was generated using EST clones (IMAGE 618035 and 367239). GST-ACBD3-fusion proteins were generated following the manufacturer's instructions (Amersham Biosciences). ACBD3 antibodies were produced in guinea pig using amino acids 1–277 and 160–525 of ACBD3, respectively (Pocono Rabbit Farm and Laboratory, Pennsylvania, USA), using standard procedures. Yeast two-hybrid assays, GST copurification assays, and coimmunoprecipitation experiments were performed as previously described (Tang et al., 2005; Yang et al., 2002) with minor modifications.

Generation of Mutant Mice and Histological Analysis

ACBD3 mutant mice were generated from XE462 ES cells (http://baygenomics.ucsf.edu) through standard blastocyst injection. The *Flox-STOP-ACBD3^{Myr}* transgene was constructed by inserting a cDNA encoding ACBD3^{Myr} into the CALSL vector (Yang et al., 2004). ACBD3^{Myr} contains a myristoylation site from chick Src and a MYC epitope (MGNCLTEQKLISEEDLEFGTRE) at the N terminus. *Nestin-Cre* and *Flox-STOP-Numbl^{SYM}* mice were generated as described elsewhere (Petersen et al., 2002; K.Z. and W.Z., unpublished data). Tissue preparation, BrdU labeling, immunostaining, in situ hybridization, TUNEL staining, and image processing were as previously described (Petersen et al., 2002, 2004; Zhong et al., 1996) with minor modifications. Antibodies used include: rabbit anti-Cleaved Caspase-3 (Cell Signaling), rabbit anti-ERp72 (Calbiochem), rabbit anti-Giantin (Covance), and rabbit anti-GM130 (a gift from Graham Warren). Noon of the day of vaginal plug was designated as E0.5. Animal use in this study was approved by the Institutional Animal Care and Use Committee at Yale University.

Quantification of Progenitor and Neuron Numbers

BrdU-labeled progenitor cells were quantified as previously described (Petersen et al., 2004) with minor modifications. To count the number of motor neurons in the developing spinal cord, thoracic regions of E10.5 control and mutant embryos were used, and Islet1-positive cells on both sides of the ventral horn on each section were counted. For each control and mutant strain, two sections each from three individual embryos were counted. The total number from each embryo was divided by two, and the resultant number was defined as the average per unit and used for statistic analysis (Microsoft Excel and SAS program).

Transgenic Overexpression in *Drosophila*

To generate transgenic flies, the cDNA encoding ACBD3^{Myr}, ACBD3$^{Myr-\Delta C}$, or m-Numb$^{\Delta 7-23}$ was cloned into the pUAST vector and injected into embryos as described elsewhere (Tang et al., 2005). ACBD3$^{Myr-\Delta C}$ is identical to ACBD3^{Myr} but lacks the C-terminal 154 amino acids. *numb^{RNAi}* flies and those expressing Numbl^{SYM} were generated previously (Tang et al., 2005; Zhong et al., 1997; H.T. and W.Z., unpublished data). *H* flies were obtained from the Bloomington Stock Center (BL-517). *Scabrous-Gal4* was used to drive transgene expression (Tang et al., 2005).

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/1/163/DC1/.

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